DEPARTMENT OF THE AIR FORCE
AIR FORCE RESEARCH LABORATORY
WRIGHT-PATTERSON AIR FORCE BASE OHIO 45433

7 June 2001

MEMORANDUM FOR US EPA
NCEA (MD-52)
RTP, NC 27711
ATTN: ANNIE M. JARABEK

FROM: Rebecca Clewell
AFRL/HEST
Operational Toxicology Branch
2856 G St, Bldg 79
Wright-Patterson AFB, OH 45433-7400

SUBJECT: Consultative Letter, AFRL-HE-WP-CL-2001-0009, Comparison of AFRL/HEST Study Protocol for Effects of Perchlorate Exposure via Drinking Water During Pre- and Postnatal Development versus a Similar Study Conducted by Argus Research Laboratories, Inc.

1. This letter describes the manner in which the studies were chosen for use in the development of the PBPK models described in the previous Consultative Letters AFRL-HE-WP-CL-2001-0006, Physiologically-Based Pharmacokinetic Model for the Kinetics of Perchlorate-Induced Inhibition of Iodide in the Pregnant Rat and Fetus, and AFRL-HE-WP-CL-2001-0007, Physiologically-Based Pharmacokinetic Model for the Kinetics of Perchlorate-Induced Inhibition of Iodide in the Lactating and Neonatal Rat. This letter also provides a preliminary analysis (Attachment 1) conducted by a third party investigator, Denny Reed of Operational Technologies, Dayton, OH, detailing the known differences in study design between Argus Research Laboratories, Inc. (Primedica, 2001) and AFRL/HEST (Yu, 2001), in an effort to determine the cause of the variation seen in the reported data.

2. The drinking water studies performed by AFRL/HEST for the gestational and lactational transfer of perchlorate were utilized in the development of PBPK models. These studies were designed for use in the kinetic modeling and therefore provided much of the needed information, such as perchlorate concentrations in maternal serum and tissues as well as fetal and neonatal serum, skin and gut. This study contains the most comprehensive data set currently available for perchlorate distribution during the perinatal period. Therefore, it was the best option for use in parameterizing the models.

3. It had been suggested that the Argus effects study could be used in validation of the previously mentioned PBPK models. To that end, the maternal, fetal and neonatal serum

samples from the Argus laboratories were analyzed for perchlorate, where sufficient serum volume was available after hormone analysis. Perchlorate analysis was performed by AFRL/HEST for all samples (Argus and HEST) with the same method, instrument and chemist, in order to minimize error. However, significant differences were seen in the perchlorate concentrations of the serum samples provided by Argus Laboratories versus those obtained in-house (see Attachment 1). Thus, the Argus samples were not used for validation of the PBPK models for the following reasons.

a. AFRL/HEST provided the most comprehensive data for perchlorate distribution in gestation and lactation.

b. The study design for the AFRL/HEST was more conducive for use in model development. For example, culling of pups was performed on PND 2, resulting in a standardized litter size of 8 pups (4 male and 4 female, whenever possible) for the PND 5 and PND 10 groups. The Argus study, however, culled the litters to 8 pups (4 male and 4 female) on PND 5, using the extraneous pups for the measurement of PND 5 serum. This method leaves room for greater variation in pup exposure for the first five days of lactation. AFRL/HEST was also able to pool neonatal serum by sex, allowing exploration of the possible sex differences in the neonatal perchlorate kinetics as well as hormone effects. Argus serum samples were pooled by litter.

c. Like the perchlorate levels, the hormone effects seen in the Argus samples were lower than those measured in the AFRL/HEST samples (Primedica, 2001; Yu, 2001). This trend, in which the Argus samples showed less serum perchlorate and diminished hormonal changes from perchlorate exposure, suggests inherent differences in the two studies.

d. The reason for the observed differences in the study was not determined in spite of efforts by the authors and an independent investigator. Without this necessary information, it is impossible to change the conditions in the model to account for the differences in the two studies. Therefore, the inability of the model to predict the data would not provide any more information about the reason for these differences.

e. The authors believe sufficient model validation was provided through the use of subsequent acute perchlorate kinetics studies, as well as iodide inhibition studies and iodide kinetics studies, which were all simulated with the model. Additionally, the structure of the model was supported by the similarity of kinetic parameters used across gender and age (Clewell, 2001a; Clewell, 2001b; Merrill, 2001).

4. In response to the questions proposed in the report by Mr. Reed, the following statements have been made.

a. Of the listed ingredients in the animal diet, none are known to interfere with perchlorate analysis. The technique for analyzing perchlorate involves the use of ion chromatography. This method is only able to detect anions. Cations, metals and lipophilic substances would not interfere with the detection of the perchlorate anion. Although fluoride concentrations were found to be quite different in the two diets, this anion is known to have a much lower

retention time than perchlorate and, therefore, would not interfere with the quantitation of perchlorate.

b. Mr. Reed suggested that the differences in the diets may affect the perchlorate dosing by changing the behavior of the rats. For example, saltier food would cause the rats to increase water consumption. This is quite possible. However, both the Argus and AFRL/HEST studies accounted for this possibility by measuring the water consumption and body weights of the rats daily. The perchlorate drinking water dosing concentrations were calculated from this information. The actual doses received by the rats are shown in Table 1 (Attachment 2).

5. In addition to those differences described in the evaluation conducted by Mr. Reed, the following issues have been noted by the others.

a. It is not known whether the ingredients listed would interfere with the kinetic behavior of perchlorate. For example, other anions, such as chloride, are also known to bind in the serum in the same manner as perchlorate. Therefore, if these anions were present in higher concentration in one of the diets, it is possible that the perchlorate anion would be displaced from serum albumin, which could result in lower measured values for perchlorate serum levels.

b. The dosing solutions prepared by Argus Laboratories were based on the molecular weight of the salt, ammonium perchlorate, as opposed to the perchlorate anion alone. The AFRL/HEST dosing solutions were based on the perchlorate anion. This results in a difference in concentration of the dosing solutions, which can be accounted for by applying a factor of 0.846 to the calculation of the actual dose concentrations used in the Argus study. The values given in Table 1 (Attachment 2) have been adjusted for this difference in molecular weight.

c. AFRL/HEST dosing solutions were consistently higher than those used in the Argus study. At PND 10, the AFRL/HEST dosing solutions were as much as 46% higher than those used by Argus, when corrected for the difference in molecular weight and the adjusted doses used by Argus to account for the changing water consumption and weight gain during pregnancy and lactation. This difference in doses may be a significant cause for the disparity seen in the serum perchlorate levels measured between the two studies. It does not appear to account for the all of the differences, such as the factor of 4 seen in the serum perchlorate concentration of dams dosed with 0.1 mg/kg-day, or the factor of 7 seen in the serum of PND 10 pups exposed to perchlorate from dams dosed with 0.1 mg/kg-day.

6. For further information, please contact me by phone: (937) 255-5150 ext. 3141, fax: (937) 255-1474 or e-mail: rebecca.clewell@wpafb.af.mil.

REBECCA A. CLEWELL
Operational Toxicology Branch

Attachments:
1. Denny Reed: Differences in Serum Perchlorate Concentrations, Argus vs. HEST Studies
2. Table 1. Actual Perchlorate Dose Received by Maternal Rats in Argus and HEST Studies on GD 20, PND 5 and PND 10
3. References

1st Ind, AFRL/HEST 7 June 2001

MEMORANDUM FOR US EPA
ATTN: MS. ANNIE JARABEK

This letter report has been coordinated at the branch level and is approved for release.

RICHARD R. STOTTS, DVM, PhD
Branch Chief
Operational Toxicology Branch
Human Effectiveness Directorate

OPTECH

OPERATIONAL TECHNOLOGIES
CORPORATION

MEMORANDUM FOR: **Rebecca Clewell**

FROM: Denny Reed

DATE: 19 April 2001

SUBJECT: Differences in Serum Perchlorate Concentrations, Argus vs. HEST Studies

1. Review of Protocols. Using the information that I received from you on March 29, 2001, including your notes regarding the similarities and differences between the Argus and HEST protocols, I developed the following summary table:

PHARMACOKINETICS OF PERCHLORATE IN PREGNANT RATS/FETUSES AND LACTATING RATS/NURSING PUPS

Argus Study	HEST Study
340 mated female (SD)IGS rats	402 pregnant female Sprague-Dawley rats
Source: Charles River Labs	Source: Charles River Labs
Weight: 200 to 225 grams upon receipt	Weight: 240 to 270 grams upon receipt
Age: at least 60 days old	Age: appropriate for weight
Drinking water: R.O. deionized ad libitum	Drinking water: R.O. deionized ad libitum
Diet: certified rodent #5002 from PMI Nutrition, Int'l	Diet: certified rodent chow #5000 from Purina Mills
Perchlorate dosage: 0, 0.01, 0.1, 1.0 or 30.0 mg/kg-day	Perchlorate dosage: 0, 0.01, 0.1, 1.0 or 10.0 mg/kg-day
Perchlorate concentration: adjusted weekly for body weight	Perchlorate concentration: held constant throughout
Perchlorate dosing begun 2 weeks before mating	Perchlorate dosing begun on GD2 (2 days after mating)
Pups shared nursing time with 12-13 other pups until PND10	Pups culled to 8 (4 male and 4 female) at PND2
Male and female rats shared drinking water during cohabitation	No cohabitation period
Blood drawn on dams via interior vena cava post sacrifice	Blood drawn on dams via vena cava while unconscious
Pup blood collected following decapitation and pooled per litter	Pup blood collected following decapitation and pooled by sex
Rats individually housed in S.S. wire-bottom cages	Rats individually housed in polycarbonate shoebox w/cellulose fiber bedding
Room temperature maintained between 64 and 79 degrees F	Room temperature maintained between 70 and 79 degrees F
Room humidity maintained between 30 and 70 percent	Room humidity maintained between 35 and 65 percent
Room air 99.97% HEPA filtered with 10 changes per hour	Room air HEPA filtered with 10 to 15 changes per hour
Blood collected in serum separator tubes to yield ~ 1125 ul	Serum obtained from blood after 15 minute centrifugation at 3000 rpm

The information contained in the above table clearly indicates that there were many similarities between the two studies, including the rat species used, their source and their living conditions. However, it is also evident that the two rat populations did not eat the same diet and the Argus rats were exposed to ammonium perchlorate in their drinking water for approximately 16 days longer than the HEST rats.

2. Summary of differences in serum perchlorate concentrations. The differences in serum levels between the two studies can be summarized as follows:

- Lactation (PND10), 0.1 mg/kg-day dose group

Group	Pup Serum	Dam Serum
Argus	0.038	0.09
HEST	0.284	0.358
Δ	7.5	4.0 [difference factor]

- Pregnancy (GD21), 0.1 mg/kg-day dose group

Group	Fetal Serum	Dam Serum
Argus	0.028	0.045
HEST	-	0.188
Δ		4.2 [difference factor]

- Pregnancy (GD21), 1.0 mg/kg-day dose group

Group	Fetal Serum	Dam Serum
Argus	0.265	0.428
HEST	-	0.706
Δ		1.6 [difference factor]

Ms. Joan Dollarhide (TERA) noted that some of the differences in serum levels between Argus and HEST, particularly at the low dose level (0.1 mg/kg-day), are due to different ways of culling and the fact that the male and female pups were pooled together in the Argus study.

3. Analysis of Difference Factors. The differences in serum perchlorate concentrations are much lower at the 1.0 mg/kg-day dosage level than they are at the 0.1 mg/kg-day dosage level. However, the differences at the lower dosage level are probably due to more factors than the difference in culling and pooling techniques between the two studies. Consequently, I have concentrated my efforts on answering the four questions discussed below.

a. Question 1. Is there a difference in drinking water consumption between the rat populations involved in the two studies?

A drinking water consumption data analysis provided the following summary statistics:

PHARMACOKINETICS OF PERCHLORATE STUDY
Daily Drinking Water Consumption (ml) - Dams

	ARGUS			HEST		
Endpoint	PND10	GD21	GD21	PND10	GD21	GD21
Dosage	0.1 mg/kg	0.1 mg/kg	1.0 mg/kg	0.1 mg/kg	0.1 mg/kg	1.0 mg/kg
Mean	57.0	47.8	45.0	36.1	34.6	35.5
Standard Deviation	16.7	10.0	9.6	11.3	9.0	9.0
Minimum	14.0	24.0	17.0	10.0	3.8	1.6
Maximum	114.0	78.0	78.0	86.4	67.0	68.3
Total Volume	8209.0	14259.0	13096.0	8093.3	8588.3	8812.2
Avg. per capita	51.3	42.4	39.0	36.1	34.1	35.0
Observations	144	298	291	224	248	248
Population	16	16	16	8	14	14

As shown in the above table the Argus rats, on average, consumed about 40 percent more water than the HEST rats. There was also a significant difference between the minimum and maximum water consumption values for the two studies, as noted in the above table.

b. Question 2. Is drinking water consumption proportional to body weight, as was assumed by both study groups?

An analysis of drinking water consumption data for the Argus rats, PND10 endpoint, dosage of 0.1 mg/kg-day of perchlorate (column 1 in the above table) indicates that water consumption was proportional to body weight. However, the proportionality factor was not constant for either study group. The ratio of body weight to water consumption weight for the lightest rats (263 grams) was about 15:1. For the heavier rats (i.e., >350 grams) the ratio of body weight to water consumption weight was about 3:1. Consequently, as their body weight increased, the rats' daily water consumption increased by a factor of 5 or more during the study period.

Analysis of drinking water consumption data for the HEST rats, PND10 endpoint, dosage of 0.1 mg/kg-day of perchlorate (column 4 in the above table) provided a similar result to the Argus study. For the lightest rat, the ratio of body weight (181 grams) to water consumption weight (10 ml) was 18:1. For the heaviest rat (398 grams), the ratio was just under 5:1 (398/86). Although the HEST rats consumed less water in proportion to their body weight than the Argus rats, the relationship between body weight and water consumption was similar between the two studies, as shown below.

HEST Study, Body Weight/Water Consumption	
Dosage Level 0.1 mg/kg to PND10	
Mean	8.023961094
Standard Error	0.092679825
Median	7.88101983
Mode	7.522193211
Standard Deviation	1.37466395
Sample Variance	1.889700977
Kurtosis	12.4428516
Skewness	1.813434654
Range	13.5100463
Minimum	4.609953704
Maximum	18.12
Sum	1765.271441
Count	220

Argus Study, Body Weight/Water Consumption	
Dosage Level 0.1 mg/kg to PND10	
Mean	5.876637658
Standard Error	0.170355367
Median	5.310344828
Mode	4.818181818
Standard Deviation	2.044264406
Sample Variance	4.179016961
Kurtosis	13.44313543
Skewness	3.046676637
Range	15.67067004
Minimum	3.115044248
Maximum	18.78571429
Sum	846.2358227
Count	144

c. Question 3 Is there a significant difference in drinking water quality between the two studies?

Analytical data for the control/blank drinking water used in the HEST study are not available. Consequently, there is insufficient information available to ascertain any significant differences in drinking water quality between the two studies. There are also insufficient data available to determine if one or more constituents in either drinking water source could account for the major differences in serum perchlorate concentrations, particularly at the 0.1 mg/kg-day dosage level.

With respect to the measured perchlorate concentrations in the dosing solutions used in both studies to achieve the 0.1 mg/kg-day target level, the differences are relatively small. Analysis of the drinking water consumption, body weight and dosing concentration data for both studies at the 0.1 mg/kg-day target level, for the PND10 endpoint, indicates that the rats were overdosed in both studies. The average dosage for the Argus rats was about 0.12 mg/kg-day, with a standard deviation of 0.03 mg/kg-day. The average dosage for the HEST rats was about 0.107 mg/kg-day, with a standard deviation of 0.017 mg/kg-day. Over the full range of available data for both studies, the calculated dosage for the Argus rats ranged from a low of 0.037 mg/kg-day to a high of 0.225 mg/kg-day. In the HEST study, the calculated dosage ranged from a low of 0.046 mg/kg-day to a high of 0.181 mg/kg-day. The summary statistics are as follows:

HEST Study, Perchlorate Dosage Statistics	
Target Dosage Level, 0.1 mg/kg to PND10	
Mean	0.106744936
Standard Error	0.001185495
Median	0.105823881
Mode	0.110871919
Standard Deviation	0.017583731
Sample Variance	0.000309188
Kurtosis	2.544831744
Skewness	0.827034207
Range	0.13488639
Minimum	0.04602649
Maximum	0.18091288
Sum	23.48388593
Count	220

Argus Study, Perchlorate Dosage Statistics	
Target Dosage Level, 0.1 mg/kg to PND10	
Mean	0.128465702
Standard Error	0.00255409
Median	0.131818182
Mode	0.145283019
Standard Deviation	0.03064908
Sample Variance	0.000939366
Kurtosis	1.248822168
Skewness	-0.126865487
Range	0.187453552
Minimum	0.037262357
Maximum	0.224715909
Sum	18.4990611
Count	144

d. Question 4. Are there any significant differences in assay between #5000 feed used in the HEST study and #5002 feed used in the Argus study?

The chemical composition of the #5000 feed used in the HEST study is very similar to the chemical composition of the #5002 feed used in the Argus study. However, the concentrations of constituents in the two feeds are significantly different. Using >25% as a benchmark, the HEST diet is significantly higher in fat content (50 to 65% higher) than the Argus diet. The HEST diet also contains nearly twice as much cholesterol and Vitamin K as the Argus diet. On the other side of the ledger, the Argus diet contains much more linoleic and linolenic acids, acid detergent fiber, lactose, chromium and folic acid than the HEST diet.

The significant differences in constituent concentrations between the HEST and Argus diets could account for some of the differences in water consumption rates that were observed between the two studies. The differences in constituent concentrations between the two diets could also account for some of the differences in serum perchlorate concentrations, if one or more of the chemical constituents present in the feed interferes with the perchlorate analysis. A summary of constituent concentrations that differed by more than 25 percent is shown below.

Rodent Diet Assay	#5000-HEST	#5002-ARGUS	DELTA	% Diff.
Glycine%	1.23	0.86	0.37	30.0813
Taurine%	0.02	0.01	0.01	50
Fat (ether extract)%	6.5	4.5	2	30.76923
Fat (acid hydrolysis)%	7.5	5.1	2.4	32
Cholesterol, ppm	280	150	130	46.42857
Linoleic Acid%	1.37	2.15	-0.78	-56.93431
Linolenic Acid%	0.09	0.16	-0.07	-77.77778
Total Saturated Fatty Acids%	2.51	0.86	1.65	65.73705
Total Monosaturated Fatty Acids%	2.32	1.14	1.18	50.86207
Acid Detergent Fiber%	4	5.9	-1.9	-47.5
Lactose%	0.39	1.11	-0.72	-184.6154
Fluorine, ppm	19.1		19.1	100
Cobalt, ppm	0.4	0.6	-0.2	-50
Chromium, ppm	1.4	2	-0.6	-42.85714
Carotene, ppm	4	5.6	-1.6	-40
Vitamin K (total), ppm	3.2	0.4	2.8	87.5
Menadione (added), ppm	2.9	0	2.9	100
Riboflavin, ppm	5	8	-3	-60
Folic Acid, ppm	3	4	-1	-33.33333
Biotin, ppm	0.2	0.13	0.07	35
Vitamin D3 (added), IU/gm	3.3	2.2	1.1	33.33333

Constituent concentration difference > 25%

Among the more notable differences in constituent concentrations between the two feeds is the higher lactose concentration and the apparent absence of fluorine in the Argus feed. The presence of menadione in the HEST feed and its total absence in the Argus feed may also be noteworthy.

In addition to the similarities and differences between the two studies discussed above, there is another potentially important difference. The Argus rats were dosed with perchlorate in their drinking water for a much longer duration than the HEST rats. As noted in the protocol comparison chart presented on page one of this memo, the Argus rats were exposed to perchlorate during the two-week pre-cohabitation period. In the HEST study, perchlorate exposure did not begin until GD2 (two days after mating). Consequently, the Argus rats were dosed with perchlorate in their drinking water for about 16 days longer than the HEST rats. At the lower dosage levels (i.e., 0.01 and 0.1 mg/kg-day), this "perchlorate preconditioning" may have caused some metabolic adjustments that contributed significantly to the lower serum perchlorate levels detected in the Argus rats/pups.

Although the actual cause(s) for the large differences in serum perchlorate concentrations that were recorded between the Argus and HEST dams/pups may never be known, it is evident from the above analysis that there were significant differences between the two studies. On average, the Argus rats consumed approximately 40 percent more water than the HEST rats. The Argus and HEST rats consumed significantly different concentrations of chemical constituents in their feed. The presence or absence of some of these constituents may have interfered with the serum

perchlorate analysis. It is also possible that the "perchlorate preconditioning" involved in the Argus study significantly lowered the serum perchlorate concentrations in the blood streams of the dams/pups.

4. Recommendations. As noted above, some of the differences in constituent concentrations between the #5000 diet used in the HEST study and the #5002 diet used in the Argus study may have contributed to the differences in serum perchlorate analysis. It may be useful to have the dietary differences reviewed by LTC Brinkley, the veterinary pathologist assigned to AFRL/HEST. It may also be useful to have the dietary differences reviewed by an analytical chemist who is familiar with the serum perchlorate analysis process. Perhaps the combination of these additional reviews and the other differences noted between the two studies (e.g., the significant differences in drinking water consumption between the HEST and Argus dams) will provide a reasonable explanation for the differences in serum perchlorate concentrations in dams and pups.

cc:
Dr. Mattie, AFRL/HEST
Teri Sterner, OPTECH
Dr. Lurker, OPTECH

Table 1. Actual Perchlorate Dose Received by Maternal Rats in Argus and HEST Studies on GD 20, PND 5 and PND 10

Day	Target dose (mg/kg-day)	Actual dose AFRL/HEST (mg/kg-day)	Actual dose Argus Laboratories (mg/kg-day)	% difference
GD20	0.01	0.0096	0.00846	12%
GD20	0.1	0.0913	0.07614	17%
GD20	1.0	0.995	0.82908	17%
PND5	0.01	0.015	0.00846	44%
PND5	0.1	0.139	0.10998	21%
PND5	1.0	1.37	1.09134	20%
PND10	0.01	0.0158	0.00846	46%
PND10	0.1	0.156	0.09306	40%
PND10	1.0	1.52	1.06596	30%

References

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